A Portable Cryo-Plunger for On-Site Intact Cryogenic Microscopy Sample Preparation in Natural Environments

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ABSTRACT We present a modern, light portable device specifically designed for environmental samples for cryogenic transmission-electron microscopy (cryo-TEM) by on-site cryo-plunging. The power of cryo-TEM comes from preparation of artifact-free samples. However, in many studies, the samples must be collected at remote field locations, and the time involved in transporting samples back to the laboratory for cryogenic preservation can lead to severe degradation artifacts. Thus, going back to the basics, we developed a simple mechanical device that is light and easy to transport on foot yet effective. With the system design presented here we are able to obtain cryo-samples of microbes and microbial communities not possible to culture, in their near-intact environmental conditions as well as in routine laboratory work, and in real time. This methodology thus enables us to bring the power of cryo-TEM to microbial ecology. *Microsc. Res. Tech. 00:000–000, 2011.*

INTRODUCTION

The first cryogenic transmission electron microscopy (cryo-TEM) grids (i.e., thin films of unsupported, vitreous ice) were obtained 30 yr ago. Initially, the imaging targets were relatively large biological objects such as viruses and bacteria. Considerable expertise and craftsmanship in cryo-grid preparation and technological progress in transmission electron microcopy (TEM) instruments have extended the range of application of this uniquely powerful technology to the study of biological macromolecules with near atomic level resolution (Stahlberg and Walz, 2008), organic compounds (Frederik and Sommerdijk, 2005) and near-intact bacteria (Milne and Subramanian, 2009). A whole range of experimental steps related to cryo-TEM sample preparation, data acquisition, and data processing have become highly standardized and automated. Most of the techniques and automation in cryo-sample preparation for "Single Particle" cryo-TEM have been readily adopted in cryo-electron tomography (cryo-ET) of intact cells and viruses.

Obtaining vitreous ice with solutions and suspensions "trapped" in a state nearly identical to the liquid phase, and without the artifacts of crystallization, was a goal unsuccessfully pursued for a long time. The turning point was achieved by Brüggeller and Mayer (1980) when they realized the main obstacle had been the size of the target bulk solution. Micrometer-scale liquid droplets could be frozen by spraying from a jet into amorphous, vitreous ice. Soon after, Dubochet and McDowall (1981) imaged vitreous droplets of pure water by TEM. These initial "cryo-grids" were prepared by spraying the water onto continuous thin carbon TEM grids with a nebulizer as they were "plunged" into ethane or propane at ~80° K. They applied their technique to aqueous suspensions of biomolecules (Dubochet et al., 1982) and imaged cryo-sections of bacteria cultures (Dubochet et al., 1983). The first cryo-TEM grid, essentially consisting of a thin film of an aqueous suspension spanning the free holes of a TEM grid frozen as amorphous ice, was achieved by Adrian and Dubochet (1984), and modern, routine cryo-TEM was thus started.

While all fundamental issues underlying good preservation in the near-intact or "near-native" state are conceptually the same from small biomolecules through biopolymers and intact bacterial cells and viruses, the demands across this range of samples can vary greatly. As recognized by Dubochet et al. (1985), the thin film of aqueous suspension vitrified in cryo-TEM samples is self-stabilizing and the simplest cryo-system designs gave the best results for suspensions of viruses and cells. The steps in preparing such samples are straight-forward: a sample droplet is placed on a cryo-TEM grid ("holey" or "lacey" carbon support), mounted in tweezers; most of the liquid is blotted off; the grid is very quickly submerged into liquid ethane at approximately its freezing point (90° K; the ethane is held in a liquid nitrogen bath). Cryo-TEM grids are designed to provide areas of unsupported vitreous ice; these are formed by surface tension of the aqueous suspension on the grid during blotting. The method con-

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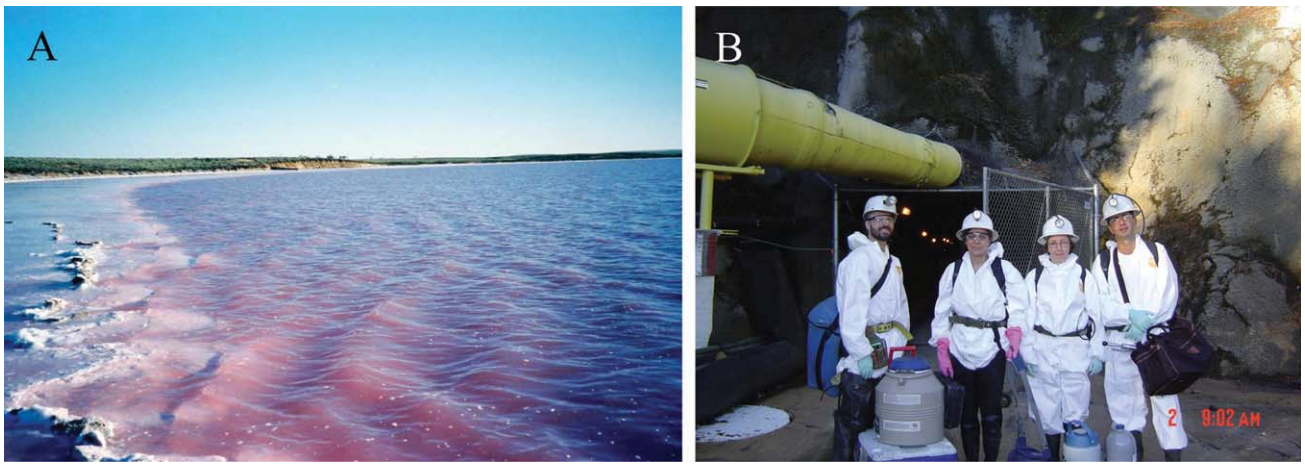

Fig. 1. Natural environments rich in extremophile microbial communities. **A**: Shores of Lake Tyrrell, Victoria, Australia. This is a hypersaline lake and the pink color is due to photosynthetic bacteria. **B**: Entrance to the IMM, in Richmond, CA. The tunnels contain numerous ponds and streams of AMD rich in biofilms. The goal is to obtain cryogenic TEM samples directly on-site at these and similarly "out of ordinary" locations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sists of forming a thin layer of the suspension and cooling it in the vitreous state under conditions such that the sample remains in a given intact (or near-intact) state.

To obtain the highest signal-to-noise from low dose cryo-TEM images, one sample-specific parameter to optimize is the thickness of the layer formed by the suspension. If the thickness approaches the largest dimension (diameter) of the target object, the result is an orientation constraint and forced surface interactions, possibly causing sample degradation and thus should be avoided. In addition, surface effects at the air-water interface must be avoided. In the case of biological macromolecules and complexes with dimensions in the range of ~5–20 nm (order of ~10 nm), the vitrified suspension should be only slightly thicker than the macromolecules dimensions, in general <50 nm. The very large surface to volume ratio means that evaporation can be critical, even on time-scales of milliseconds. In a typical thin film preparation under room conditions, temperature ~20°C and humidity ~40%, in one second the film looses 40 nm of thickness (Frederik and Storms, 2005). In addition, the temperature of the sample would change as a consequence of evaporation. Changes in the osmolarity can be in the order of 2× or more within 1–2 s for film thickness in the order of ~100 nm. These dramatic changes in pH, sample and solute concentration can result in structural changes, associations, dissociations, and collapse of the structures. Nonetheless, great progress in cryo-TEM was achieved utilizing custom-made, "in-house" plunge freezing devices with manual sample blotting. However, this methodology requires a high level of user dexterity (Iancu et al., 2007). This user experience, including the perception of changes in local climate to achieve robust artifact-free reproducibility, is hard to acquire, transmit, and consistently replicate. Moreover, the use of cryo-TEM in the study of delicate, vulnerable samples, such as weakly associated macromolecules, micelles and lipid vesicles, and in nanochemistry in general, often requires more sophisticated control of the blotting process (Frederik and Sommerdijk, 2005).

For these reasons, efforts were made to provide systems for sample preparation (cryo-TEM grid blotting and vitrification) with controlled humidity and temperature for the study of hydrated organic, biological, and colloidal dispersions. Controlled environmental vitrification systems (CEVS; Bellare et al., 1988) have become the standard for the study of cryo-TEM samples and the basis for the "Vitrobot™" patented by the University of Maastricht and licensed to FEI (Frederik and Sommerdijk, 2005; Iancu et al., 2007). This and similar devices such as the Gatan CP3 (Gatan, Pleasanton, CA) and the Leica EM GP (Leica Microsystems GmbH, Wetzlar, Germany) freezing systems are reported to produce consistent, reproducible, high quality results. In theory, sample-specific protocols with computerized parameters allow new students and researchers to obtain the same consistent results at different times or places.

This sophisticated automation of cryo-TEM sample preparation has significantly improved the ability to study the most delicate nano-chemistry systems. However, the cryo-TEM field has lost some of the ability to understand how to obtain optimal samples at the other extreme: the coarser level of whole bacteria, microbial communities, and environmental samples. For whole bacteria samples, the vitreous ice surrounding the bacteria must be at least slightly thicker than the diameter of the bacteria (if they are not to be deformed). This means, in general, around and above 500 nm. The evaporation rate will only decrease the thickness by ~40 nm in 1 s, <10%. Issues such as solute concentration and osmolarity, and change in temperature, are second order if the user is proficient. Equivalent samples can thus often be obtained using the simplest "in-house" or "home-made" cryo-plunger and the Vitrobot™.

There are many advantages in having a simple, custom-made "in-house" cryo-plunger when dealing with more complex microbial samples. With environmental microbial samples (e.g., Baker et al., 2010; Comolli et al., 2009, 2011), containing extra-cellular polymeric substances (EPS), sediment nanoparticles, or even

Fig. 2. Fully mechanical and portable cryo-plunger. **A**: Model view. The tweezers are attached to the piston in a recess in the piston rod with a spring-loaded latch, easy to access and operate. The piston is driven by a spring. Different spring strengths as well as gravity can be chosen. The Dewar can be easily exchanged and is within a cylindrical shield made of transparent acrylic to decrease condensation during small intervals in the operation. **B**: A brass cup at the center of the Dewar holds liquid ethane or propane in a bath of liquid nitrogen; an aluminum ring holds cryo-boxes to store grids, readily accessible to the tweezers. **C**: The portable cryo-plunger mounted on a tripod for easy use in the field. Half of the transparent acrylic shield has been removed for better viewing. The base fits a cylindrical Dewar for liquid nitrogen and ethane, which can hold six cryo-boxes for temporary grid storage. The Vitrobot™ Dewar also fits and can be used in our tool and is shown at the tripod base in the photograph of panel (C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

large structures to which cells are attached (Comolli et al., 2011), we find the impossibility of fine-tuning the blotting procedure due to the occluded view of the grid in an automated freezing device to be one important inconvenience. Oftentimes the drop applied to the grid is not perfectly homogenous, or becomes inhomogeneous while the grid is in the plunging tweezers. It is a great advantage to be able to see the sample and choose the blotting angle as well as the blotting pressure. Another related aspect is that as the blotting in the Vitrobot™ is done on the two sides of the grid simultaneously, we find a difficulty in controlling just how much of the micrometer-size extracellular material interacts with, and becomes attached to, the blotting paper. Work with some systems, such as extremophiles directly obtained from the environment, requires high speed and dexterity because of the acidity (pH below 1.0) and high ionic strength (mM to M) of the solutions. Cryo-grids of acid mine drainage (AMD) biofilm samples (Baker et al., 2010; Comolli et al., 2009; Knierim et al., 2011) degrade carbon-coated Formvar copper grids within seconds. In addition, each grid is unique from all others due to the variability of biofilm fragments and residues that are taken by the pipette each time. Thus, for optimal results no fixed set of parameters can be automatically used for all grids; user judgment and experience are critical. For these more complex, coarse and thicker samples, going back to basics and using custom-made "in-house" simple cryo-plungers seems to ensure higher quality. Certainly in this range of samples Dubochet's initial judgment holds true: "of all the various designs of cryosystems explored, the simplest give the best results" (Dubochet et al., 1985).

The Need for a Small, Economical, and Transportable Cryo-Plunger

The majority of the issues surveyed above can probably be overcome, either by implementing small modifi-

cations or customizing the use of the automated cryo-plungers for each case. This may take more time and effort than the acquisition of dexterity in the operation of a simple, "in-house" cryo-plunger. However, there remains the need to cryo-plunge in the environment, often in remote sites such as those shown in Figure 1 without electricity, benches, or easy access. The simple but modern, light-weight, fully mechanical, and portable cryo-plunger designed for our environmental samples and presented in this report addresses these problems. The relatively small size and light weight of the device allow it to be safely mounted on a tripod and used in difficult terrains, such as the interior of a mine or the shores of salt lakes. It can also be transported to other laboratories, such as synchrotron facilities, for on site preparation of samples for correlated characterization. Finally, for users with experience this and other "in-house" cryo-plungers produce the highest quality results for microbial samples. Because it costs more than an order of magnitude less than the available automated plungers, it can be adopted by many laboratories around the world that cannot afford to purchase an automated unit, or that would rather develop craftsmanship to optimize their budget towards different needs.

MATERIALS AND METHODS

Construction of the Portable Cryo-Plunger

Three-dimensional models of the portable cryo-plunger were generated using Computer Aided Design (CAD) software. Machine shop drawings were generated using 2D CAD software. All pieces were fabricated in a standard machine shop from aluminum, Plexiglas and standard, commercially available hardware. The Dewar was built from a common medium density foam block. A complete device will be available upon special order requests. For more detailed design drawings and additional information about licensing this technology, please contact LBNL Technology Transfer and Intellectual Property Management office at http://www.lbl.gov/Tech-Transfer.

Cryo-TEM Specimen Preparation

Aliquots of 5 μL were taken directly from lake water (Lake Tyrrell, Sea Lake, in Western Victoria, Australia); groundwater and groundwater concentrates (DOE's Uranium Mill Tailings Site in Rifle, Colorado); and AMD biofilms of various stages of growth (Iron Mountain, CA), and placed on lacey carbon grids (cat no. 01881, Ted Pella, Redding, CA). The Formvar support was not removed from the lacey carbon. Tests were also done with various laboratory cultures and other samples (not shown). The grids were pretreated by glow-discharge and deposition of colloidal gold in the laboratory before the trips as described before (Comolli et al., 2011; Knierim and Luef, 2011). The grids were manually blotted with filter paper and plunged into liquid ethane or propane near liquid nitrogen temperature, then stored in liquid nitrogen.

Cryo-TEM Imaging

Images were acquired on a JEOL JEM–3100 FFC transmission electron microscope equipped with a FEG electron source operating at 300 kV, an Omega energy filter, a Gatan 795 2×2 K^2 CCD camera (Gatan, Pleasanton, CA), and a cryo-transfer stage. The stage was cooled with liquid nitrogen to 80 K during acquisition of all data sets. Bright field images were acquired using a magnification of 25 k× at the CCD, except those shown in Figures 6E and 6F, acquired at 50 and 70 k× at the CCD, respectively.

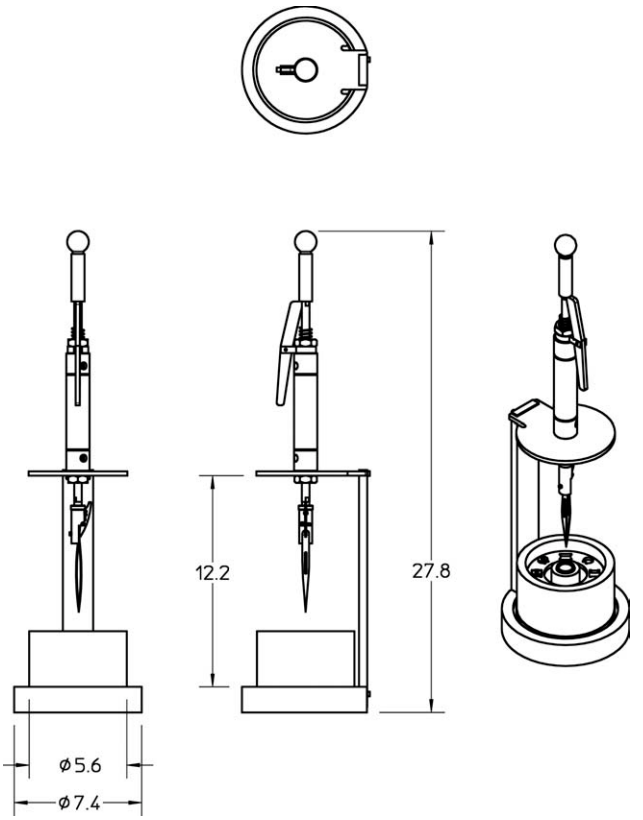

Fig. 3. Portable cryo-plunging line drawings. The scales are in inches. A complete device will be available upon special order requests. For more detailed design, drawings and additional information about licensing this technology, please contact LBNL Technology Transfer and Intellectual Property Management office at http://www.lbl.gov/Tech-Transfer.

RESULTS

A Fully Mechanical and Portable Cryo-Plunger

A simple, purely mechanical cryo-plunger was built with an aluminum frame and a latch-operated piston that carries the tweezers, Figures 2 and 3, and Supporting Information Movie S1 S1. The design reflects both some of the earliest in-house plunger designs, and features that significantly enhance stability and utility in the field. It weights 4 kg, has a height of 71 cm and is mounted on a circular base 18.8 cm diameter. Most of the weight of the device is in the base to provide stability. The base fits a Dewar made from medium density foam block, which holds the liquid nitrogen bath. The Dewar and piston can be shielded within cylindrical covers to prevent condensation and excessive liquid nitrogen evaporation during brief intervals in cryo-plunging (Fig. 2A). At the center of the Dewar, a brass cup holds the liquid ethane or propane, and in the circumference, an aluminum ring holds the grid-storage boxes (Fig. 2B). In remote locations and extreme envi-

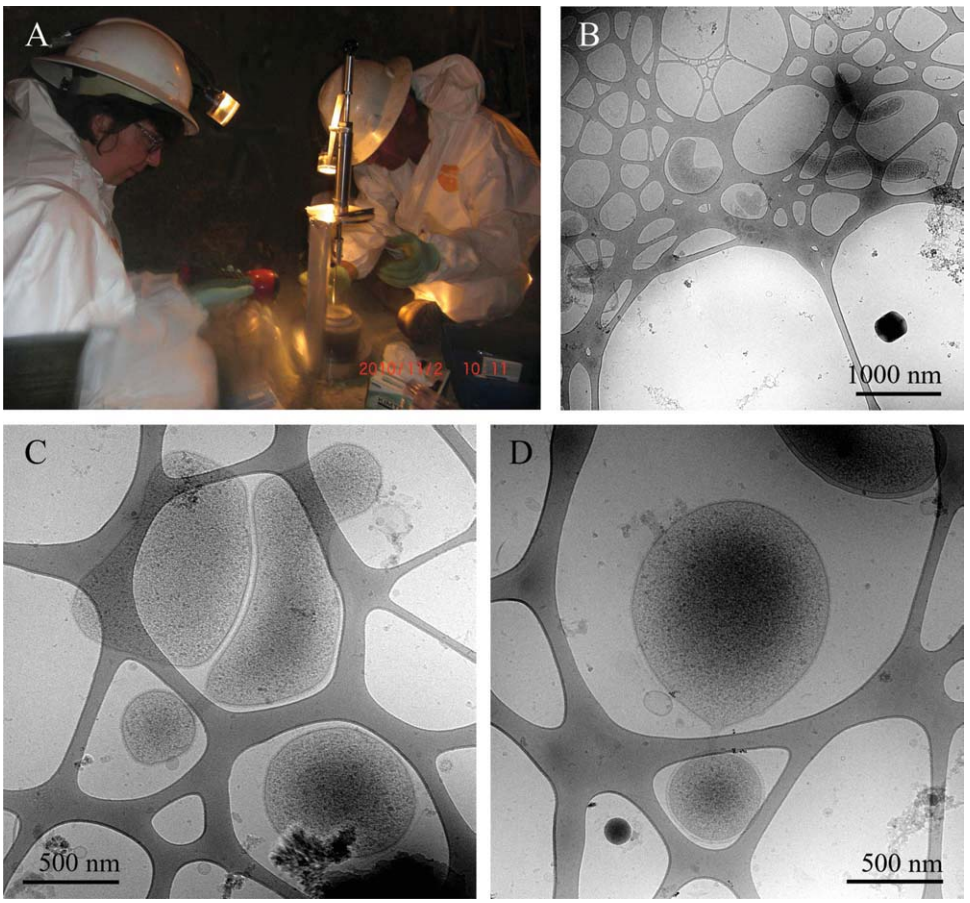

Fig. 4. Portable cryo-plunger in use and examples of results. **A**: Cryo-plunging AMD biofilm samples inside the IMM, Richmond CA. **B:** Low dose defocused diffraction cryo-TEM image of a cryo-grid made inside the IMM as shown in (A). A few different species of microorganisms can be readily recognized. The high-contrast object at the bottom-right is a mineral particle; to the right of the particle some typical extra-cellular aggregate. A relatively empty or "not crowded" area of the grid was chosen for display to illustrate the lack of ice contamination and the thin, transparent ice obtained. **C** and **D**: They are bright field images of AMD archaea. The two round organisms in (D) are of different species (very different cell wall structures) connected through a cell-to-cell bridge or "synapse", partially occluded by a carbon-coated Formvar support film (Baker et al., 2010). At the top of panel (D) a small part of a bacterium has been imaged. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ronments a commercially available propane cylinder/tank from a hardware store may be used, although this is not ideal due to impurities and uncertainty about the liquefied gas freezing point. After blotting and plunging, the tweezers are released from the piston and moved to a position to release the grid into a cryo-box slot (Fig. 2B). An aluminum cylinder at the top holds a spring, which imparts momentum to the piston. The mechanical release is a handle, held by either the right or left hand in Figure 2C. At the bottom of the base, a tripod can be screwed in, and the device placed directly on the terrain (Fig. 2C). The animation shown in Supporting Information Movie S1 S1 provides additional views of the device and the sequence of actions throughout operation. The goal of the system design is to optimize the overall efficiency and operation of a portable device.

PERFORMANCE AND APPLICATIONS

A prototype of our device was first tested directly during a field trip to Lake Tyrrell, Sea Lake, in Western Victoria, Australia. We have amply tested the finished device in the laboratory, cryo-plunging and imaging a wide range of samples side by side with an older, custom-made "in-house" device (which requires electricity and compressed air) used for decades at Donner Laboratory, LBNL (e.g., Li et al., 2002). We routinely use the new portable device on the bench for a wide range of bacterial samples and suspensions of nanoparticles, as we need to judge and adapt to samples quickly. We have also used the final version of the portable cryo-plunger preparing cryo-grids with environmental samples directly on-site, with great success, at two other locations: DOE's Uranium Mill Tailings Site in Rifle, Colorado in August and September of 2010 and 2011 (Luef et al., 2011), and inside the Iron Mountain Mine (IMM), Richmond, CA, in November of 2010, Figure 4. The required dexterity (Iancu et al., 2007) and "craftsmanship" required to operate a cryo-plunger on-site (Fig. 4A) are rewarded with good quality grids and high quality cryo-TEM images (Figs. 4B and 4C). Moreover, the acquired dexterity is a critical factor enabling the finding of novel organisms and biology within their intact environments (Fig. 4D). In situa-

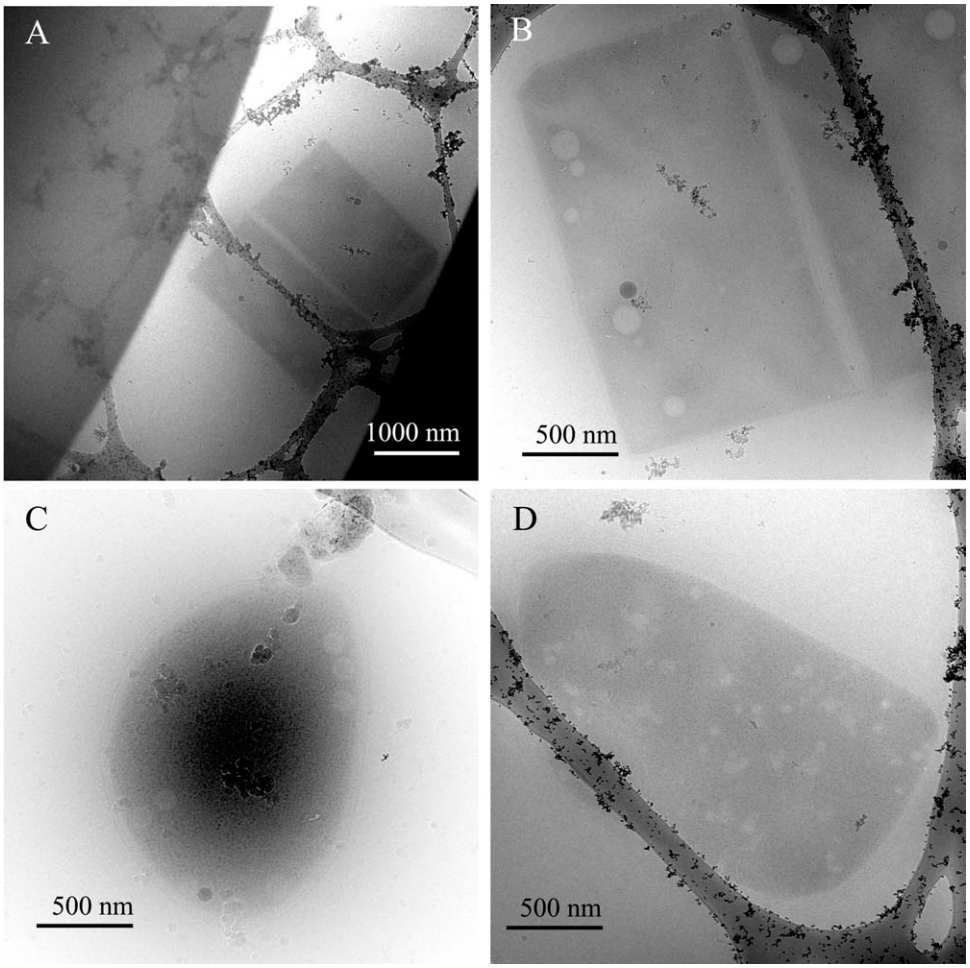

Fig. 5. Microorganisms living in hypersaline waters: cryo-TEM images from samples made in Lake Tyrrell, Victoria, Australia. **A**: Low dose defocused diffraction image of two intact *Haloquadratum walsbyi*. **B**: A bright field image of part of one of the cells shown in (A). **C** and **D**: Two un-identified microorganisms.

tions of less extreme geographically imposed constraints, such as the shores of a lake in a remote location, a small infrastructure of concentrators, pumps, and centrifuge may be in place for metagenomics and metaproteomics work. A portable cryo-plunger offers the ability to prepare cryo-samples at various stages of a cycle directly and immediately. Some organisms may be anaerobic and require instant freezing, others may be adapted to hypersaline environments, and most organisms cannot yet be cultivated in the laboratory. Even if they could be cultivated in the laboratory, we would like to observe them exactly as they are in their natural conditions. For example, cryo-plunging on-site at Lake Tyrrell, Australia, allowed us to compare cryo-TEM data of *Haloquadratum walsbyi* (Figs. 5A and 5B) with previous laboratory-based work (Burns et al., 2007) and to observe microorganisms probably never described before (Figs. 5C and 5D).

DISCUSSION

Extensive laboratory testing of cryo-grid preparations were done prior to the field trips. Donner Lab, LBNL, has had a custom-made, "in-house" plunge freezing device for several decades. This cryo-plunger was utilized to obtain the cryo-grids that provided several high-resolution tubulin structures (e.g., Li et al., 2002, and references therein) and *Caulobacter crescentus* cryo-ET data (e.g., Bowman et al., 2010). We thoroughly compared our new portable cryo-plunger, the "in-house" Donner cryo-plunger, and a Vitrobot™ throughout a period of more than a year while preparing samples for the development and calibration of a novel correlative cryo-TEM and fluorescence *in situ* hybridization (FISH) method (Knierim and Luef, 2011; several hundreds of images from dozens of cryo-plunging sessions). These samples consisted of *C. crescentus* laboratory cultures, AMD biofilms grown in a bioreactor, and AMD samples obtained from the field. Sediment samples from Rifle were also shipped to the laboratory for additional cryo-grid preparation providing as well, as a byproduct, a thorough comparison with cryo-grids made on-site.

These tests served to support the conclusion that with dexterity (Iancu et al., 2007) or "craftsmanship" simple custom-made cryo-plungers consistently provide artifact-free cryo-TEM samples. More importantly,

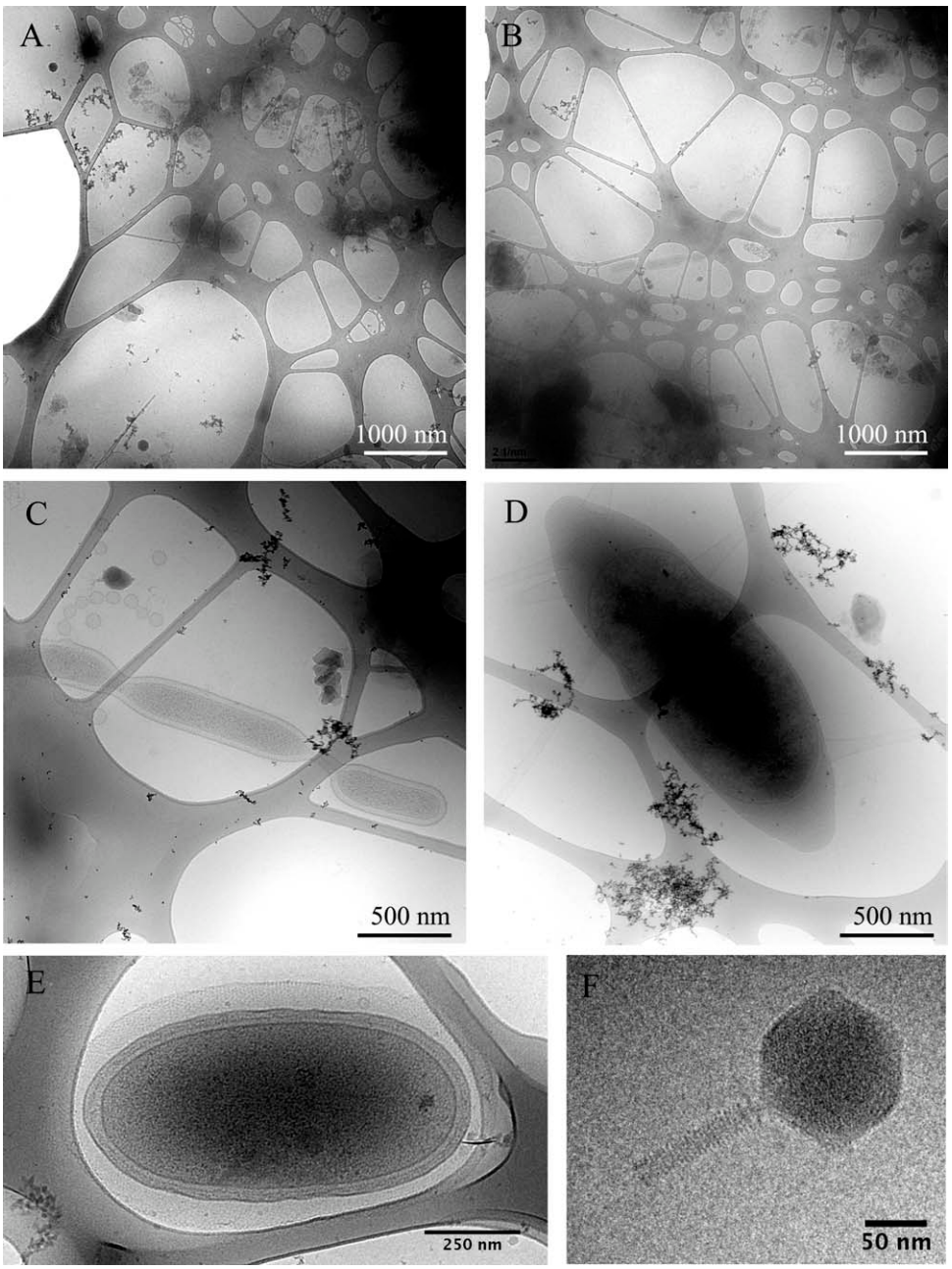

Fig. 6. Cryo-TEM images of microbial species in groundwater and sediment samples from the DOE's Uranium Mill Tailings Site in Rifle, Colorado. **A** and **B**: are low dose defocused diffraction images of a typical groundwater and sediment sample obtained for surveys and target selection. Sufficiently thin high quality ice must be achieved even though the sample is rich in sediments, as in top-right of (A) and bottom-left of (B). Several microorganisms, across a wide range of sizes are commonly found (**C**–**F**). C: Bright field image of the chain-like dividing microorganism seen in (B). D: Bright field image of a *Spinomonas maritime*-like microorganism. E: Conspicuous S-layer covering a microorganism. F: A virus, very often present in this sample. These grids contain sediments, large and small microorganisms, viruses, and a range of novel biology yet to be understood. All images shown in this figure except (F) were obtained from grids cryo-plunged in the laboratory as part of a thorough comparison of performance between laboratory and natural settings.

they allowed us to understand how to make successfully cryo-grids with complex samples directly obtained from the environment and rich in microorganisms of various sizes as well as sediments and EPS (Fig. 6). Due to the presence of sediments, minerals, and biofilm fragments of all sizes, each aliquot placed on a grid prior to plunging can be different and unique. Moreover, there is a choice and user bias in pipetting each aliquot from the heterogeneous sampling source.

We provide an extended survey of cryo-TEM grids obtained from environmental samples rich in sediments, EPS, high in ionic strength and/or very acidic and thin pieces of biofilms. For the type of natural systems described here, we routinely use our portable

cryo-plunger in the laboratory setting. We also continue to expand the range of natural microbial communities we intend to explore. Microbiologists are increasingly aware that the research on bacterial laboratory cultures alone is not sufficient. For example, even in readily cultivated strains such as *E. coli*, perhaps over 35% of the genome can be lost compared with their environmental counterparts (e.g., Fux et al., 2005), and these lost genes include structures necessary for surface attachment and infection, ideal targets for cryogenic microscopy. Only some environmental samples could be readily transported to a laboratory without sample degradation before freezing. More importantly, this device allows the study of novel bacterial and archaeal strains and communities that might never be cultivatable in the laboratory. We feel this goal will be appreciated by environmental microbiologists, geomicrobiologists, geobiochemists, and in general by scientists interested in these questions. In addition, this device will facilitate collaborative efforts of all sorts, as it can be easily transported anywhere. Thus, this lightweight, simple, and affordable cryo-plunger could be of use even for laboratories that routinely use one of the commonly used, fully automatic plungers.

In summary, the portable cryo-plunger is an essential piece of equipment enabling imaging-based analysis of microorganisms in environmental and other samples. Samples can be obtained on-site, in real time, without suffering the detrimental effects of transportation, changes in temperature, humidity, and loss of homeostasis in general. Microorganisms are thus captured in a near-intact state, within the network of interactions and in the metabolic stage determined by the local environment.

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